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DTRA-TR-06-24

TECHNICAL REPORT

The Pathogenesis and Therapy of Combined Radiation Injury

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October 2006

20061122236

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REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
00-10-2006	Technical report	

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
The Pathogenesis and Therapy of Combined Radiation Injury	DTRA01-03-D-0022
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER 468D

6. AUTHOR(S)	5d. PROJECT NUMBER
R.S. Boudagov, L.P. Oulianova and A.F. Tsyb	BD
	5e. TASK NUMBER EB
	5f. WORK UNIT NUMBER DH02935

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
ITT Industries, Inc. Advanced Engineering & Sciences 2560 Huntington Avenue Suite 500 Alexandria, VA 22303-1410	DTRA-TR-06-24

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
Defense Threat Reduction Agency 8725 John J. Kingman Road MS 6201 Fort Belvoir, VA 22060-6201	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES
This work was sponsored by the Defense Threat Reduction Agency under RDT&E RMSS code B 468D D K334 BD EB 02935 25904D

14. ABSTRACT

Cytokine production by different kinds of macrophages, and serum IL-1β, TNF-α, IL-6, IL-3, and GM-CSF levels, were evaluated in a murine model of combined injury (whole body irradiation + thermal burn). The results were compared with systemic cytokine responses to radiation or burn alone. The ob-tained data suggest that increased IL-6 levels may play an important role in the pathogenesis of combined injury. Single injection of extract from Serratia marcescens (Imuvert) or synthetic trehalose dicorynomy-colate improved hematological status, enhanced the serum IL-6 levels, but did not promote survival in mice. On the other hand, heat-killed Lactobacillus acidophilus demonstrated a weak effect on cytokine response, did not improve severe hematological disorders, but none the less increased 30 days survival in mice up to 100%, versus 50% in the untreated group.

15. SUBJECT TERMS
Combined Injury   Biological Response Modifiers   Cytokine Responses   IL-6 Production

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT UNCLASSIFIED	b. ABSTRACT UNCLASSIFIED	c. THIS PAGE UNCLASSIFIED	UNCLASSIFIED	50	19b. TELEPHONE NUMBER (include area code)

CONVERSION TABLE

Conversion Factors for U.S. Customary to metric (SI) units of measurement.

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angstrom	1.000 000 x E -10	meters (m)
atmosphere (normal)	1.013 25 x E +2	kilo pascal (kPa)
bar	1.000 000 x E +2	kilo pascal (kPa)
barn	1.000 000 x E -28	meter^2 (m^2)
British thermal unit (thermochemical)	1.054 350 x E +3	joule (J)
calorie (thermochemical)	4.184 000	joule (J)
cal (thermochemical/cm^2)	4.184 000 x E -2	mega joule/m^2 (MJ/m^2)
curie	3.700 000 x E +1	*giga bacquerel (GBq)
degree (angle)	1.745 329 x E -2	radian (rad)
degree Fahrenheit	$t_k = (t°f + 459.67)/1.8$	degree kelvin (K)
electron volt	1.602 19 x E -19	joule (J)
erg	1.000 000 x E -7	joule (J)
erg/second	1.000 000 x E -7	watt (W)
foot	3.048 000 x E -1	meter (m)
foot-pound-force	1.355 818	joule (J)
gallon (U.S. liquid)	3.785 412 x E -3	meter^3 (m^3)
inch	2.540 000 x E -2	meter (m)
jerk	1.000 000 x E +9	joule (J)
joule/kilogram (J/kg) radiation dose absorbed	1.000 000	Gray (Gy)
kilotons	4.183	terajoules
kip (1000 lbf)	4.448 222 x E +3	newton (N)
kip/inch^2 (ksi)	6.894 757 x E +3	kilo pascal (kPa)
ktap	1.000 000 x E +2	newton-second/m^2 (N-s/m^2)
micron	1.000 000 x E -6	meter (m)
mil	2.540 000 x E -5	meter (m)
mile (international)	1.609 344 x E +3	meter (m)
ounce	2.834 952 x E -2	kilogram (kg)
pound-force (lbs avoirdupois)	4.448 222	newton (N)
pound-force inch	1.129 848 x E -1	newton-meter (N-m)
pound-force/inch	1.751 268 x E +2	newton/meter (N/m)
pound-force/foot^2	4.788 026 x E -2	kilo pascal (kPa)
pound-force/inch^2 (psi)	6.894 757	kilo pascal (kPa)
pound-mass (lbm avoirdupois)	4.535 924 x E -1	kilogram (kg)
pound-mass-foot^2 (moment of inertia)	4.214 011 x E -2	kilogram-meter^2 (kg-m^2)
pound-mass/foot^3	1.601 846 x E +1	kilogram-meter^3 (kg/m^3)
rad (radiation dose absorbed)	1.000 000 x E -2	**Gray (Gy)
roentgen	2.579 760 x E -4	coulomb/kilogram (C/kg)
shake	1.000 000 x E -8	second (s)
slug	1.459 390 x E +1	kilogram (kg)
torr (mm Hg, 0° C)	1.333 22 x E -1	kilo pascal (kPa)

*The bacquerel (Bq) is the SI unit of radioactivity; 1 Bq = 1 event/s.
**The Gray (GY) is the SI unit of absorbed radiation.

Summary

This work was supported by the Armed Forces Radiobiology Research Institute (AFRRI), and the Defense Threat Reduction Agency (DTRA). The authors wish to thank E. John Ainsworth, Ph.D., who has supported the planning for this project, and Dr. Glen I. Reeves, MD from AFRRI for constant support given to MRRC RAMN. Heat-killed *L. acidophilus* was kindly provided by V. Pospelova (Moscow). We are grateful to Tatiana Kliachina and Tatiana Eriomina for technical assistance, long hours of work and patience at all stages of experiments. The authors thank Dr. V. Skvortsov, Dr. G. Rott, Dr. V. Lepechin, Ms. N. Seleva, Ms. I. Sokolova, and Ms. N. Shalavkina for their assistance in this project. Lastly, it is with regret that Dr. R.S. Boudagov passed away before his research was published; Dr. Boudagov died on November 23, 2005.

Funding and contractual management support for the production and publication of this report was provided by DTRA. The editor is indebted to Dr. Paul K. Blake, Nuclear Test Personnel Review Program, DTRA, who consistantly supported the production of these reports. The agency is grateful for the report production and technical editing provided by Chris Brahmstedt of the Defense Threat Reduction Information Analysis Center (DTRIAC), as well as the valuable technical contributions and suggestions provided by William Billado and Don Alderson of DTRIAC for this report.

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Cytokine production by different kinds of macrophages, and serum IL-1β, TNF-α, IL-6, IL-3, and GM-CSF levels, were evaluated in a murine model of combined injury (whole body irradiation + thermal burn). The results were compared with systemic cytokine responses to radiation or burn alone. The obtained data suggest that increased IL-6 levels may play an important role in the pathogenesis of combined injury. Single injection of extract from *Serratia marcescens* (Imuvert) or synthetic trehalose dicorynomycolate improved hematological status, enhanced the serum IL-6 levels, but did not promote survival in mice. On the other hand, heat-killed *Lactobacillus acidophilus* demonstrated a weak effect on cytokine response, did not improve severe hematological disorders, but none the less increased 30 days survival in mice up to 100%, versus 50% in the untreated group.

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1.0 Introduction

At Hiroshima and Nagasaki, over two thirds of injured survivors had more than one type of injury (radiation, mechanical trauma, thermal burns.) At Chernobyl, of the 31 people who died from acute radiation syndrome, two thirds of these had significant thermal burns; at the higher levels of radiation exposure, almost 100% of patients had burns. Troops exposed to a nuclear weapon or explosive radiological dispersal device (RDD), or who may be required to control a major reactor accident or incident, will therefore be more likely to have combined injury (CI) rather than only radiation exposure.

The clinical course and outcome of CI are more severe than in acute radiation syndrome alone. Even thermal burns or wound trauma that would be benign by themselves are capable of decreasing the survival rate of patients exposed at minimally lethal or midlethal radiation dosages. Myelosuppression and prolonged neutropenia, induced by accidental exposure, will contribute to morbidity and complications associated with increased susceptibility to endogenous infection. Exposed patients with additional traumatic injury die during the first two or three weeks after irradiation mainly due to sepsis [12, 13, 84, 85]. Until recently, however, the mechanisms responsible for increased morbidity and mortality among those with CI were not clear. The differences between the pathogenesis of combined vs. radiation alone injury must be more clearly understood than at present.

Currently it is well known that cytokine-mediated proliferation and differentiation of quiescent hematopoietic stem cells are prerequisite for survival after life-threatening whole body irradiation. The cytokines serve as natural defenses against radiation. The endogenous production of cytokines contributes to the animal's ability to survive midlethal doses of radiation. Studies using antibodies to IL-1 receptor, TNF, IL-6, and anti-c-kit ligand antibody have demonstrated that endogenous production of these cytokines is required for mice to survive lethal irradiation [32, 53, 58, 60, 95]. The interdependence and synergistic interactions of these three key cytokines are apparently necessary to demonstrate many of their biological effects. IL-1, IL-6, and TNF stimulate production of a cascade of hematopoietic growth factors, including IL-3, G-CSF, GM-CSF, that are more restricted to hematopoietic effects [59, 61].

Unfortunately, until recently, there have been no published experimental data evaluating production of the multifunctional cytokines IL-1β, IL-6, and TNF-α by tissue macrophages after irradiation alone and combined injury. Published experimental data that simultaneously evaluate serum levels of these and more lineage-specific hemoregulatory cytokines after irradiation alone and combined injury are also missing.

At the same time, the analysis of recent publications showed that thermal injuries *per se* may induce essential changes of different cytokines. The systemic cytokine response to burns is mainly represented by IL-1β, TNF-α and IL-6. These cytokines are the most important endogenous mediators which are responsible for the post-burn mortality associated with bacterial endotoxemia and sepsis. Bone marrow, alveolar, splenic or peritoneal macrophages, primed in vitro by LPS, produced different amounts of TNF, IL-6 and IL-1 compared

with sham burned animals [25, 67, 70]. Increasing evidence shows that cells other than immune cells (hepatocytes, enterocytes) have the potential for enhanced production of TNF and IL-6 after thermal injury [65, 68, 69]. Microvascular endothelium may be a contributing source of IL-6 after thermal injury [4]. Unburned skin may be a major source of IL-6 production after thermal injury and may contribute to the physiologic alterations occurring after such injury [34]. Thermal burns cause significant changes of the serum cytokine levels that play key roles in acute inflammation, excess protein turnover and catabolism, multiple organ failure, and immunosuppression [3, 17, 23, 40, 41, 71, 80].

In other words, if combined injury occurs, burn-induced cytokine response may enter into interaction with the radiation-induced response of the cytokine network. It is hard to predict the results of such an interaction. One can expect that there will be significant differences in the types, amounts and rates of cytokine elaboration between acute radiation injury and combined injury. Therefore, one of the main objectives of this study is comparative experimental evaluation of the system cytokine response (macrophage production and serum levels) to irradiation alone, or to combined injury. When compared with corresponding hematological data (early blood system responses to radiation alone, or to combined injury), the demonstrated results would give new useful information for understanding the aggravating effects of burns on the acute radiation syndrome. This is the first study that examined the production of TNF-α, IL-1β, and IL-6 by three different populations of macrophages as well as evaluated serum TNF-α, IL-1β, IL-3, GM-CSF, and IL-6 levels from the mice subjected to irradiation or combined injury. The results of the experimental study would be especially important as a scientific basis for the future improvement of combined injury treatment in mass casualty situations.

Several in vivo studies suggest the importance of hematopoietic regulatory cytokines and growth factors, such as G-CSF, GM-CSF, IL-1, IL-3, IL-11, and TPO, for the treatment of accidental radiation-induced bone marrow aplasia. Hemopoietic growth factors and cytokines, administered therapeutically after total body irradiation, significantly improved hematopoietic recovery and increased survival of lethally irradiated animals [19, 39, 55, 63, 81, 83, 88, 89]. Therapeutic protocols that included injection of two or three types of cytokines (GM-CSF + IL-3, IL-3 + IL-6, G-CSF + IL-6, stem cell factor + IL-1 + IL-3, megakaryocyte growth and development factor + G-CSF, TPO + G-CSF, IL-11 + IL-3 etc.) demonstrated more significant acceleration of hematological status recovery in comparison with single cytokine therapy [20, 21, 24, 44, 56, 75, 91]. Therapeutic approaches focused on the evaluation of lineage-specific cytokines and the using of combinations of cytokines protocols to enhance hematopoietic recovery from irradiation have developed mainly in the USA and in the framework of international scientific collaboration [1, 14, 15]. Unfortunately, this new effective approach probably will not be used in mass casualty situations because of limited resources.

An alternative to the schemes of prolonged administration of multiple cytokines is a single injection of so-called biologic response modifiers (BRMs) that induce the endogenous expression of hematoregulatory cytokines or growth factors, stimulate multipotential hematopoietic progenitors, accelerate the recovery of hematopoietic parameters and increase survival [11, 30, 46 - 48, 52, 78].

Bacterially derived BRMs, such as a dephosphorylated derivative of the lipid A moiety of LPS, extract from *Serratia marcescens* (Sm-BRM), and synthetic trehalose dicorynomycolate (S-TDCM), differentially enhanced elevated splenic gene expression of hematoregulatory cytokines after sublethal gamma irradiation. S-TDCM, and Sm-BRM, that sustained or even enhanced irradiation-induced expression of specific cytokine genes, increased plasma CSF activity and improved survival after experimental infection [78]. A single injection of a heat-killed *Lactobacillus casei* preparation, given after irradiation, significantly enhanced granulopoiesis, increased the amount of endogenous spleen colonies as well as increased serum M-CSF activity, augmented expression of M-CSF mRNA in the liver, and increased the survival rate of lethally irradiated mice [26, 62, 86].

At the same time, S-TDCM did not increase survival of irradiated mice that also received a skin wound. Lethal gamma or mixed gamma-neutron irradiated mice inflicted with wound trauma succumbed to sepsis before augmented hematopoietic regeneration could occur. Significant increase of the survival rate was achieved only when systemic treatment with S-TDCM or gentamicin was combined with topical treatments of gentamicin cream [38, 45]. Our own experience confirms the preliminary hypothesis that not all BRMs that are recommended for treatment of acute radiation syndrome alone may be optimal therapy for patients suffering from combined injury. At least, only individual preparations from the large number of evaluated BRMs were able to significantly increase 30 day survival. It should be emphasized that these BRMs promoted survival without improvement of hematological status [5 - 7, 35, 74]. Moreover, at present nothing is known about the effects of in vivo BRMs administration on the systemic cytokine response during combined injury.

So, several BRMs that enhanced radiation-induced expression of specific cytokine genes improved hematopoietic cell recovery and prevented death after irradiation. Whether these new therapeutic remedies will be able to enhance an "appropriate" cytokine response, improve blood system status, and increase survival of mice that have been subjected to combined injury, remains to be determined. This study will analyze the responses of combined injured versus irradiated only mice to therapeutic administration of three BRMs (S-TDCM, *Serratia marcescens* extract, and heat-killed *Lactobacillus acidophilus*).

In general, the authors of this final report investigated, in a murine model of combined injury, whether: 1) macrophages from these animals produce different amounts of TNF-α, IL-1β and IL-6 compared with irradiated only mice; 2) thermal burns given to irradiated mice alter the radiation-induced blood serum level of these and other hemoregulatory cytokines; 3) the single therapeutic administration of BRMs in the early stages of combined injury corrects the blood serum level of cytokines; and 4) the single administration of BRMs improves hemopoietic recovery and increases 30 day survival from combined injury.

It was established that the levels of cytokine production by peritoneal, splenic, and bone marrow macrophages after combined injury did not differ versus acute radiation syndrome, as a rule. Burn-induced effects modified radiation-induced alteration of serum cytokine levels, and revealed a more evident increase of serum IL-6 levels after combined injury. A single injection of both S-TDCM and Sm-BRM improved the hematological status of combined injury mice and

enhanced serum IL-6 levels, but did not promote survival of the mice. Heat-killed *Lactobacillus acidophilus* revealed a relatively weak modifying effect on the serum IL-6 levels, and did not improve severe hematological disorders after combined injury. However, a single injection of heat-killed *Lactobacillus acidophilus* increased the 30 days survival up to 50% versus untreated mice.

2.0 Materials and Methods

2.1 Experimental Design and General Procedures

Male mice CBAxC57BL6 weighing 22-24 g were used for the experiments throughout this study. Animals were quarantined and acclimated to laboratory conditions for at least two weeks prior to experimentation. Mice were irradiated in the Medical Radiological Research Center's ^{60}Co gamma radiation source (Obninsk, Russia). Irradiation was performed to a total absorbed dose of 7 Gy at a dose rate of 0.45 Gy/min. Mice receiving burns only or combined injury received a full thickness thermal burn to 10% of the total body surface area (TBSA) by means of a powerful light exposure. The thermal burn was given immediately following irradiation in the combined injury subjects.

Animals were anesthetized (intraperitoneal injection of Nembutal, 2 mg/100 gm) prior to shaving the dorsum and inflicting the burn. All four mouse paws were fixed with tape to a plastic board, and the dorsal skin area shaved to the indicated area using a published formula for computing TBSA from body weight. Shaved mice, fixed to a plastic board, were placed into a box template that exposed 10% of their TBSA. The area of the box outside the open window had heat-shielding material. Six halogen lamps were positioned parallel to the exposed skin surface, 10 mm away. Light exposure time was 1.5 seconds. To reduce dehydration, immediately after induction of thermal trauma all animals received intraperitoneal resuscitation with 1.0 ml normal saline. Fluid resuscitation of burned mice, consisting of one ml saline daily, was performed until sacrifice. The thermal injuries induced caused a "dry" (coagulated) form of skin necrosis; healing took place under dry eschar, and suppurative complications or ulcerations were rare. Sham burned animals were anesthetized, shaved, and placed into a box template without exposure to light. These normal healthy mice were used as a control group.

Cytokine production (IL-1β, IL-6 and TNF-α) by peritoneal, splenic and bone marrow macrophages were studied at 3, 6, 24, 48 and 72 hours after irradiation, thermal burn or combined injury. Normal healthy animals served as a control group. Ten mice from each group were sacrificed at every indicated observation point, for a total of 200 mice. The serum levels of IL-1β, IL-3, IL-6, GM-CSF and TNF-α were measured at the same time intervals and in the same four experimental groups. Twenty mice were used at each point of observation, for a total of 400 mice. Cytokine production from cultured macrophages, and serum cytokines levels were determined by enzyme-linked immunosorbent assay (ELISA).

Peripheral blood cell values (leukocytes, platelets, and erythrocytes), bone marrow cellularity, and the number of endogenous spleen colony-forming units (CFUs) were evaluated as well as measurements of cytokine production.

2.2 Macrophages and Culture Conditions

Resident peritoneal macrophages were harvested by peritoneal lavage. After washing, macrophages were suspended in cold (+4° C) Eagle's medium supplemented with heparin (10 U/ml), gentamicin (80 μg/ml), L-glutamine (0.2 mM),

and 10% bovine serum. Spleens were harvested and homogenized in 5 ml cold complete Eagle's medium by means of manual glass tissue homogenizer. Bone marrow cells were obtained by flushing both femora with 1 ml of Eagle's medium per femur to obtain sufficient cells for in vitro cytokine production assays. Macrophages were harvested in aseptic conditions. Sterile technique was used throughout all cell culture experiments.

After centrifugation and repeated washing of each cell suspension with cold Eagle's medium, complete Eagle's medium was added to the cell sediments. Suspended cells were seeded into plastic Petri dishes (150 x 15 mm) and were allowed to adhere for 2.5 hr at 37° C in 5% CO_2 – 95% air atmosphere. To remove nonadherent cells, the dishes were washed three times by pre-warmed Eagle's medium. The concentration of peritoneal and bone marrow macrophages from each animal was adjusted to 0.5 x 10^6 /ml, and splenic macrophages to 2 x 10^6 /ml, in RPMI 1640 medium supplemented with gentamicin (80 μg/ml), L-glutamine (0.2 mM) and 10% heat-inactivated bovine serum. For determination of the production of IL-1, IL-6, and TNF, 2 ml of macrophages suspension were added to each well of a 6-well culture plates. After adherence, macrophages were incubated at 37° C for 24 hr in the presence of 5% CO_2 in an automatic digital CO_2 incubator (Flow Laboratories, England). Three of the six wells contained cells and medium alone (evaluation of spontaneous cytokine production), and the next 3 wells contained cells, medium and LPS from *Escherichia coli* 055:B5 (1 μg/ml, evaluation of in vitro LPS-stimulated cytokine production). Splenic macrophages were cultured in the presence of 2 μg/ml LPS from *E.coli*. The numbers of macrophages used for incubation, doses of LPS for in vitro macrophage stimulation, and duration of cell culture were selected based on review of the literature [16, 18, 22, 25, 66, 79]. Macrophages from each mouse were cultured separately. At the end of incubation, cell-free culture supernatants were collected and stored at -40° C until assayed for cytokine concentration. Each supernatant sample was evaluated for IL-1β, IL-6 and TNF-α levels detection.

2.3 Cytokine Assays

Concentrations of IL-1β, IL-6 and TNF-α in cell-free culture supernatants were measured using commercial matched antibody pair and standards for quantitation of mouse cytokines (ELISA MiniKits, "Endogen", USA). 96-well immunoplates were used. Each test sample and the diluted standards were added to immunoplates in duplicate. The assays of IL-1β, IL-6, and TNF-α were performed according to the manufacturer's instructions. Optimal working concentration of biotin-labeled detecting antibody, and coating antibody, as well as HRP-conjugated streptavidin dilution, were determined beforehand by using "checkerboard matrix" titration experiments in accordance with Endogen's "Guide to custom ELISA development with matched antibody pairs and supporting reagents". Phenylenediamine substrate was used for the detection system. The wavelength 492 nm was set at the ELISA reader (Uniplan-M "Picon" Inc.). This reader was supplied by a special programmed device for automatic construction and calibration of a standard curve construction, calculation of cytokine concentration, and printing out the final results on paper.

Commercially available matched antibody pairs for quantization of mouse IL-

1β, IL-3, IL-6, TNF-α and GM-CSF ("Biosource", "R&D", "Endogen", USA) were used to measure the serum concentration of cytokines. Blood samples were obtained and allowed to clot at room temperature for two hours. The serum was then separated by centrifugation. Serum samples were frozen and stored prior to assay. Assays were performed after twofold dilution of pooled sera (two mice/ pool) using buffer solutions recommended by manufacturer's instructions.

The ELISA tests had sensitivities of 50 pg/ml for IL-1β, 50 pg/ml for IL-3, 25 pg/ml for IL-6, 7.8 pg/ml for GM-CSF, and 50 pg/ml for TNF-α.

2.4 Reagents, Cell Culture and ELISA Supplies

RPMI 1640 medium, lipopolysaccharide from *Escherichia coli* 055:B5, Strep-tavidin-Peroxidase, Thimerosal, and bovine serum albumin were obtained from Sigma Chem. Co. (St. Louis, USA). TRIS buffer was obtained from Serva (Germany), and Tween-20 from Ferak (Germany). Nembutal, gentamicin, L-glutamine, bovine serum, Eagle's medium, and phosphate buffered saline were purchased from PanEco (Moscow, Russia), and phenylendiamine substrate from Dia-M (Moscow, Russia).

There were 6-well culture plates (Costar Corp., MA, USA), 96-well immuno-plates ("96F Nunc Immunoplate I", Denmark), polystyrene conical tubes with screw caps (Becton Dickinson, USA), micro test tubes and Eppendorf Eurotips (Eppendorf, Germany) were used.

2.5 Hematology

Bone marrow cellularity, endogenous CFUs and blood cells counts (leukocytes, platelets, and erythrocytes) were evaluated at the aforementioned observaiton points for cytokine production study. A number of nucleated cells in the bone marrow suspensions was determined after centrifugation and repeated washing of cell suspension with cold Eagle's medium. All measurements of peripheral blood were made using an automatic hematology analyzer MINOS STX (France).

2.6 Biological Response Modifiers

Three BRMs (synthetic trehalose dicorinomycolate, extract from Serratia marcescens, and heat-killed *L. acidophilus*) were studied for their effects on the serum levels of cytokines after burn only, radiation only, or combined injury. A group of healthy normal mice, injected with BRM, served as control. Eight mice were required at each point of observation: 3, 6, 24, 48 and 72 hours, for a total of 160 for each BRM. The total for all three BRMs plus a control group of non-treated animals was 640 mice. A single injection of synthetic trehalose di-corinomycolate (STDM, 200 µg, intraperitoneally), or Imuvert (commercially available preparation of extract from *Serratia marcescens*, 100 µg, intraperi-toneally) was performed one hour after irradiation, burns, or CI. Heat-killed *Lactobacillus acidophilus* (LA) was injected immediately after irradiation, burns, or CI (0.1 ml, 10^8 cells/ml, subcutaneously). In addition, a fourth group of mice was injected with saline only as the control BRM. Doses for the STDM and Imuvert were selected based on the review of literature published from AFRRI [38, 45; 78]. The concentration selected for the third BRM is based on our previous experience with this agent [8, 87].

In addition, 240 mice were used to evaluate BRM therapeutic effects on the survival rate, number of endogenous CFUs, bone marrow and blood cell recovery after combined injury (80 mice for each of the 3 BRMs). 80 mice with combined injury were studied as the control (untreated) group.

Aliquots of STDM emulsion for injections were prepared in accordance with manufacturer's instruction (Ribi ImmunoChem. Res., Inc). Lyophilized samples of Imuvert were resuspended with water for injection immediately prior to use. Lyophilized samples of heat-killed *L. acidophilus* were dissolved in saline *ex tempore* too.

2.7 Statistics

Cytokine concentrations in cell-free supernatants from cultured macrophages or in blood serum, peripheral blood cell values (leukocytes, platelets, erythrocytes), bone marrow cellularity, and the number of endogenous spleen colony-forming units (CFUs) were calculated as the mean ± SEM. Differences between compared groups were analyzed using Student's t-test, one-way ANOVA (the software package MicroCal Origin, version 3.0), and Mann-Whitney U-test. Survival data for mice in experimental groups during 30-day periods after combined radiation injury (treated with BRMs and untreated) were compared by using Fisher's test. A p value of <0.05 was used as the level of statistical significance.

All animal experiments were approved by the Armed Forces Radiobiology Research Institute's Animal Care and Use Committee.

3.0 Results

3.1 In Vitro Production of Cytokines by Different Populations of Macrophages Following Radiation, Thermal or Combined Injury

The results of IL-6 ELISA assays of the culture supernatants of peritoneal macrophages, incubated with or without addition of LPS to the culture medium, are shown in Table 1 and are expressed as percentage of control values. (When absolute values were determined, LPS-stimulated peritoneal macrophages produced larger amounts of IL-6 than that produced by unstimulated cells). Radiation and combined injury revealed a moderate increase of IL-6 production within the first six hours, and caused a more prominent second phase of cytokine elevation at two and three days after exposure. There were no significant differences between radiation and combined injury groups in the release of IL-6 by peritoneal macrophages at any time point after exposure (both with and without LPS stimulation). In spite of the comparatively much higher IL-6 elevation in 24 h after burn alone, peritoneal macrophages of mice that were burned and irradiated did not release detectable amounts of IL-6 at the indicated time.

Table 1. Effects of Radiation, Combined Injury, and Burn on Murine Peritoneal Macrophage IL-6 Production (pg/ml).

Group	3 hr	6 hr	24 hr	48 hr	72 hr
Spontaneous Cytokine Production					
Radiation	183 ± 16*	143 ± 16*	ND	557 ± 92*	292 ± 6*
Combined injury	201 ± 22*	166 ± 28*	ND	416 ± 38*	301 ± 6*
Burn	90 ± 11	133 ± 18	1326 ± 385*	554 ± 114*	181 ± 7*
Control	100 ± 10	100 ± 12	100 ± 21	100 ± 28	100 ± 20
LPS-stimulated Cytokine Production					
Radiation	162 ± 13*	125 ± 14	ND	583 ± 71*	237 ± 15*
Combined injury	155 ± 13*	136 ± 12*	ND	549 ± 47*	256 ± 12*
Burn	105 ± 13	100 ± 11	782 ± 163*	575 ± 53*	177 ± 12*
Control	100 ± 12	100 ± 12	100 ± 19	100 ± 17	100 ± 16

* $p < 0.05$ with respect to control values. ND - not detectable levels.

No detectable levels of TNF-α were revealed in the cell-free culture supernatants of peritoneal macrophages following radiation, thermal or combined injury, as well as in the control group.

In vitro production of IL-1β was detected and elevated only at 72 h after radiation (231 ± 77 pg/ml), combined injury (197 ± 92 pg/ml), and burn (173 ± 29 pg/ml) in the LPS-stimulated peritoneal macrophages cultures studied. Differences between radiation and combined injury groups were not statistically significant.

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Neither radiation alone nor combined injury enhanced in vitro production of IL-1β, TNF-α and IL-6 by splenic macrophages. Also in the control group (normal healthy mice), the levels of all three cytokines in culture supernatants were lower than the sensitivity limits of the ELISA. However, splenic macrophages produced increased amounts of IL-6 at 24 h after thermal burn (500 ± 247 pg/ml, non-stimulated cells response; 568 ± 227 pg/ml, LPS-stimulated response). This transitory activation of the splenic macrophages, harvested from burned mice, was simultaneously accompanied with LPS-stimulated elevation of IL-1β. Concentration of IL-1β in the culture supernatants increased from an undetectable level (control value) up to 142 ± 34.6 pg/ml.

IL-6, IL-1β, and TNF-α were not detected in the culture medium of non-stimulated or LPS-stimulated bone marrow macrophages following radiation, thermal or combined injury at 3, 6, 24, and 48 hours. Bone marrow macrophages, incubated in the presence of LPS at the culture medium, revealed an increased IL-6 production only at 72 h after radiation (255 ± 65 pg/mL) and burn alone (517 ± 77 pg/mL); control value was determined as 103 ± 20 pg/mL. There was no detectable level of IL-6 in the cell culture supernatants of LPS-stimulated bone marrow macrophages followed combined injury.

3.2 Serum Cytokine Response Following Radiation, Thermal or Combined Injury

Concentrations of IL-1β, TNF-α, IL-6, GM-CSF and IL-3 in the serum of normal healthy mice were below the ELISA test sensitivity limits. As the experimental study showed, gamma irradiation did not increase cytokine levels in the systemic circulation. The absolute majority of irradiated mice did not revealed detectable amounts of IL-1β, TNF-α, GM-CSF and IL-3 in their serum samples at 3, 6, 24, 48 and 72 hours after exposure.

Neither burn only nor combined injury induced an elevation of serum IL-1β, IL-3, GM-CSF or TNF-α levels within the first three days after injuries. The evaluated blood samples that were obtained from burned or combined injury mice did not revealed detectable amounts of these cytokines.

Significant differences were revealed in the IL-6 serum levels after irradiation or combined injury. Combined injury induced time-dependent changes of IL-6 concentration among a much larger number of animals in comparison with mice that were irradiated only (serum samples with detectable amounts of IL-6 were more frequent in combined injury mice). The results of IL-6 concentration measurements are presented in Table. 2. As shown, combined injury induced the most expressed and protracted increase of serum IL-6 levels.

Table 2. Effects of Radiation, Combined Injury, and Burn on Serum IL-6 Levels (pg/ml).

Group	3 hr	6 hr	24 hr	48 hr	72 hr
Radiation	69 ± 16.7	53 ± 10.6	ND	ND	ND
Combined injury	317 ± 76.0	337 ± 88.7	61 ± 6.2	194 ± 16.6	111 ± 10.9
Burn	269 ± 82.0	174 ± 35.3	ND	ND	ND
Control (no injury)	ND	ND	ND	ND	ND

ND - not detectable levels.

3.3 The Early Blood System Response Following Radiation, Thermal or Combined Injury

Radiation only caused an initial increase of white blood cells (WBC) at 6 h after exposure followed by development of leukopenia up to 72 h (Table. 3). Burn trauma was accompanied by a significant increase in the WBC count at 3-6 hr after injury along with simultaneous development of hemoconcentration. Hematological disorders after combined injury included initial leukocytosis and hemoconcentration, as well as early developing leukopenia. It should be stressed that combined injury caused a more rapid decrease in WBC and platelet numbers than did radiation only. No differences were noted in the degree and rate of development of the first bone marrow devastation phase after radiation alone or after combined injury.

Table 3. Hematological Status of Mice Following Radiation, Thermal, and Combined Injury.

Group	3 hr	6 hr	24 hr	48 hr	72 hr
White Blood Cells (x 10^3/mm^3)					
Radiation	6.8 ± 0.89	9.8 ± 0.60*	3.7 ± 0.69	1.7 ± 0.37*	1.4 ± 0.26*
Combined Injury	8.7 ± 0.60*	7.8 ± 0.73	5.0 ± 1.0	1.0 ± 0.26*	0.4 ± 0.10**
Burn	10.9 ± 0.60*	10.8 ± 0.60*	6.2 ± 0.66	5.8 ± 1.00	5.5 ± 0.79
Control	5.5 ± 0.91	5.5 ± 0.91	5.8 ± 0.92	5.3 ± 0.90	5.0 ± 0.72
Red Blood Cells (x10^6 / mm^3)					
Radiation	5.4 ± 0.21	5.8 ± 0.10	6.0 ± 0.10	5.6 ± 0.05	5.3 ± 0.44
Combined Injury	6.3 ± 0.09**	5.6 ± 0.22	5.9 ± 0.08	6.0 ± 0.20	5.1 ± 0.20
Burn	6.2 ± 0.09*	5.9 ± 0.20	6.3 ± 0.44	5.9 ± 0.13	5.8 ± 0.08
Control	5.7 ± 0.07	5.7 ± 0.07	5.8 ± 0.32	5.6 ± 0.15	5.6 ± 0.25
Hemoglobin (g/dl)					
Radiation	13.1 ±0.51	12.6 ± 0.16	12.3 ± 0.19	11.3 ± 0.34	12.3 ± 0.37
Combined Injury	14.9 ± 0.23**	12.1 ± 0.49	12.4 ± 0.20	12.7 ± 0.54	13.5 ± 0.44
Burn	13.3 ± 0.40*	12.7 ± 0.29	14.4 ± 0.22	11.3 ± 0.43	14.3 ± 0.18
Control	12.3 ± 0.08	12.3 ± 0.08	13.0 ± 0.56	12.1 ± 0.22	13.3 ± 0.49

Table 3. Continued

Group	3 hr	6 hr	24 hr	48 hr	72 hr
Platelets (x 10^3 /mm^3)					
Radiation	284 ± 14	252 ± 6	226 ± 13	287 ± 13	243 ± 8
Combined Injury	255 ± 11	250 ± 17	197 ± 25	217 ± 12**	179 ± 8**
Burn	235 ± 12	259 ± 13	231 ± 32	311 ± 15	282 ± 9
Control	260 ± 5	260 ± 5	249 ± 20	317 ± 13	231 ± 16
Bone marrow cells (x 10^6/femur)					
Radiation	11.7 ± 1.42*	11.5 ± 1.28*	5.6 ± 0.75*	2.4 ± 0.31*	0.4 ± 0.05*
Combined Injury	13.4 ± 1.34*	10.1 ± 1.02*	5.0 ± 0.59*	2.1 ± 0.17*	0.4 ± 0.05*
Burn	15.0 ± 1.56	15.9 ± 1.25	13.5 ± 1.4*	15.1 ± 0.89*	13.1 ± 1.71*
Control	18.6 ± 1.64	18.6 ± 1.64	18.1 ± 1.64	20.3 ± 0.67	18.2 ± 1.44

* $p < 0.05$ with respect to Control values

** $p < 0.05$ with respect to Control values and Radiation group

3.4 The Effects of BRMs on the Serum Cytokine Response Following Radiation, Thermal or Combined Injury

Not one of the three examined BRMs enhanced release of the hemoregulatory cytokines IL-3, GM-CSF, and TNF-α into the serum of normal healthy mice. Concentration of these cytokines remained lower than ELISA test sensitivity limits also in all examined serum samples at 3, 6, 24, 48, and 72 hours following radiation, thermal or combined injury.

Single injections of STDM and heat-killed *L. acidophilus* (LA) did not increase IL-1β levels in the systemic circulation of normal healthy mice. IL-1β remained as not detectable also in the murine serum samples of the three examined experimental groups ("burn alone", "radiation alone", "combined injury"). Only Imuvert application (the commercially available preparation of extract from *Serratia marcescens*) exerted transitory influence upon the serum levels of IL-1β (Table. 4). As shown, there were no statistical significant differences between the "radiation" and "combined injury" groups after Imuvert administration ($P_t > 0.05$).

Table 4. Concentration of IL-1β (pg/ml) in the Serum of Mice Treated with Imuvert.

Group	3 hr	6 hr	24 hr	48 hr	72 hr
Radiation	433 ± 28.2	264 ± 35.3	ND	ND	ND
Combined injury	490 ± 109.4	145 ± 53.5	ND	ND	ND
Burn	262 ± 39.2	188 ± 37.8	ND	ND	ND
Control (no injury)	435 ± 74.4	83 ± 8.1	ND	ND	ND

ND - not detectable level.

The most evident capacity of BRMs to enhance endogenous serum cytokine responses following radiation, thermal burn, and combined injury was revealed when serum IL-6 level was measured (Table 5).

Table 5. Concentration of IL-6 (pg/ml) in the Serum of Mice Treated with Different Biological Response Modifiers.

Group	3 hr	6 hr	24 hr	48 hr	72 hr
Control(No treatment)	ND	ND	ND	ND	ND
Control + LA	500 ± 124	458 ± 17	ND	ND	ND
Control + IMUVERT	7870 ± 238	2210 ± 188	63 ± 11	ND	ND
Control + STDM	1693 ± 107	3884 ± 163	375 ± 68	112 ± 36	ND
Radiation (No treatment)	69 ± 17	53 ± 11	ND	ND	ND
Radiation + LA	227 ± 43	527 ± 8	ND	ND	ND
Radiation + IMUVERT	9930 ± 70	8221 ± 132	93 ± 12	82 ± 35	ND
Radiation + STDM	1765 ± 550	4424 ± 609	751 ± 48	302 ± 24	96 ± 24
Combined injury (No treatment)	317 ± 76	337 ± 89	61 ± 6	194 ± 17	111 ± 11
Combined injury + LA	754 ± 54	489 ± 31	55 ± 8	151 ± 17	267 ± 82
Combined injury + IMU-VERT	9641 ± 121	8627 ± 304	448 ± 24	182 ± 19	154 ± 16
Combined in-jury + STDM	1139 ± 159	3255 ± 372	3130 ± 749	789 ± 248	187 ± 30
Burn (No treat-ment)	269 ± 82	174 ± 35	ND	ND	ND
Burn + LA	715 ± 56	607 ± 54	ND	ND	ND
Burn + IMU-VERT	9214 ± 115	8700 ± 242	227 ± 19	183 ± 33	225 ± 74
Burn + STDM	2826 ± 328	5590 ± 228	733 ± 100	85 ± 11	56 ± 17

ND - not detectable level.

Heat-killed *L. acidophilus* subcutaneous injection induced moderate increase of IL-6 concentration in the serum of control mice (no injury) within the first 3-6 hours, up to 450-500 pg/ml on average, by comparison with not detect-

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able levels of IL-6 in the healthy control mice (untreated group). A roughly similar transitory enhancing effect of LA to the serum IL-6 level was observed in the burn, radiation, and combined injury groups. Slightly increased levels of IL-6 at 24, 48, and 72 hours after combined injury and LA injection did not differ statistically from the response of IL-6 in the combined injury group without treatment at the observation points stated above.

A single intraperitoneal administration of Imuvert to normal mice caused very strong serum IL-6 levels elevation at 3 and 6 hours (7,870 ± 238 pg/ml, and 2,210 ± 188 pg/ml, respectively), followed by a sharp decrease in the cytokine amount 24 hours after drug injection. A rise of IL-6 concentrations in the sera of irradiated, burned, and combined injury mice, treated with Imuvert, was even larger than the previous control values ($p < 0.05$). The differences between the three studied groups (radiation, burn, and combined injury) at 3 and 6 hours after Imuvert injection were not statistically significant.

The concentration of IL-6 increased in 3 hours and reached its maximum in 6 hours (3884 ± 163 pg/mL) after a single injection of STDM to the normal control mice. STDM maintained moderate serum IL-6 activity also at 24 and 48 hours after administration. The burned animals' response to STDM at 3 and 6 hours was the most significant as compared with the controls (healthy mice which received STDM). From all the studied groups, only the combined injury mice kept up the peak level of IL-6 (more than 3000 pg/ml) 24 hours after STDM injection.

Thus, all three BRMs induced maximal levels of IL-6 during the first 3-6 hours after injection. Imuvert proved to be the most active IL-6 levels "enhancer". Heat-killed *L. acidophilus* showed relatively weak activity among the experimentally evaluated BRMs. STDM was characterized by an intermediate activity (with respect to Imuvert and LA) within the first 3-6 hours after injection but exerted rather more prolonged stimulatory influence on the serum IL-6 level. It is necessary to emphasize that Imuvert and STDM given after combined injury maintained increased serum IL-6 levels at 24 and 48 hours, even despite the relatively high cytokine levels endogenously produced by combined injury itself (without BRM administration).

3.5 Effects of BRMs on Mouse Survival Rate and Blood System Recovery After Combined Injury

Survival of control mice with combined injury amounted to an average of 50% (Fig.1). The best therapeutic result was achieved when heat-killed *L. acidophilus* was injected; 100% of animals survived until the 30th day. Single administration of Imuvert or STDM did not significantly modify the survival rate of control (untreated) mice with combined injury. There were no statistically significant differences of mean survival time in mice with combined injury treated with Imuvert (12.1 ± 3.35 days) or STDM (11.9 ± 1.24 days), as compared with the untreated control group (10.9 ± 0.61 days).

STDM and Imuvert showed significant ($P_t < 0.01$) increase of endogenous CFUs number, up to 6.7 ± 1.00 and 11.6 ± 3.58 respectively, as compared with the untreated control group (2.6 ± 0.58). Injection of LA did not cause any difference in CFU production (3.6 ± 1.11).

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STDM accelerated the intensity of bone marrow cellularity recovery rate. On the contrary, mice given Imuvert showed a one-week delay of the recovery rate. At the same time, LA did not modify the recovery rate of bone marrow that was related to combined injury (Table 6).

Table 6. Effects of Different Biological Response Modifiers on the Bone Marrow Recovery after Combined Injury.

Group	7 days	14 days	21 days	30 days
CI (control)	1.0 ± 0.70	16.3 ± 1.27	22.5 ± 1.59	19.6 ± 1.28
CI + LA	1.0 ± 0.09	16.3 ± 1.88	22.9 ± 2.41	21.9 ± 2.34
CI + STDM	$2.0 \pm 0.25*$	$22.2 \pm 3.29*$	$15.0 \pm 1.77*$	17.7 ± 1.98
CI + Imuvert	1.3 ± 0.17	$11.8 \pm 1.26*$	20.4 ± 3.25	19.2 ± 3.15

* $p < 0.05$ with respect to CI (control) values.
The results are number of bone marrow cells $(x\ 10^6)$ per femur.
Healthy mice (no injury, no treatment) - 19.7 ± 0.80

Results of blood cells recovery after combined injury and BRM administration are presented in Table 7. As one can see, not one of the examined BRMs improved severe blood cell deficiency during the most crucial clinical phase (seven days after combined injury). Moreover, injection of STDM decreased the white blood cell count even more, owing to granulocyte deficiency. Injection of STDM caused wide individual differences of white blood cell recovery rates 14 and 21 days after combined injury. However, the general tendency was for the rates to increase more than in the combined injury control group.

The recovery phases of leukopenia and granulocytopenia in the surviving mice (14, 21 and 30 days observation points) was more monotonous in the "combined injury + LA" and "combined injury + Imuvert" groups than in the STDM group. All three BRMs caused a similar degree of moderate and transitory increase of erythrocytes (14 days after combined injury) and platelets (21 days after combined injury) with respect to untreated mice. However, these peculiarities, demonstrated among subpopulations of surviving mice, are most likely not important factors in determining the prognosis in combined injury.

Table 7. Effects of Different Biological Respose Modifiers on Blood Cell Recovery After Combined Injury.

Group	7 days	14 days	21 days	30 days
White Blood Cells (x10³ /mm³)				
Healthy mice (no injury, no treatment) - 6.4 ± 0.58				
CI (control)	0.4 ± 0.05	5.4 ± 0.63	6.5 ± 0.55	4.7 ± 0.37
CI + LA	0.4 ± 0.04	4.9 ± 0.74	4.4 ± 0.68*	4.0 ± 0.75
CI + STDM	0.2 ± 0.03*	7.1 ± 1.10	15.7 ± 6.29	4.5 ± 0.61
CI + Imuvert	0.4 ± 0.05	4.8 ± 0.60	5.3 ± 0.56	4.6 ± 0.49
Granulocytes (x10³ /mm³)				
Healthy mice (no injury, no treatment) - 1.80 ± 0.17				
CI (control)	0.24 ± 0.04	1.55 ± 0.20	3.34 ± 0.31	1.43 ± 0.15
CI + LA	0.17 ± 0.02	1.76 ± 0.30	1.85 ± 0.43*	1.62 ± 0.38
CI + STDM	0.09 ± 0.03*	3.87 ± 0.69*	8.18 ± 4.23	1.33 ± 0.22
CI + Imuvert	0.23 ± 0.03	2.29 ± 0.39	2.25 ± 0.23*	1.75 ± 0.21
Red Blood Cells (x10⁶ / mm³)				
Healthy mice (no injury, no treatment) - 5.3 ± 0.10				
CI (control)	4.3 ± 0.14	4.4 ± 0.15	6.2 ± 0.13	5.3 ± 0.06
CI + LA	4.5 ± 0.26	5.0 ± 0.18*	6.2 ± 0.24	5.3 ± 0.07
CI + STDM	4.7 ± 0.13	5.1 ± 0.25*	6.0 ± 0.33	5.3 ± 0.17
CI + Imuvert	4.4 ± 0.28	5.1 ± 0.28*	5.6 ± 0.15*	4.9 ± 0.17*
Platelets (x10³ /mm³)				
Healthy mice (no injury, no treatment) - 311 ± 35.4				
CI (control)	52 ± 13.9	290 ± 17.8	238 ± 14.8	267 ± 9.1
CI + LA	35 ± 11.0	319 ± 21.9	302 ± 18.4*	232 ± 23.3
CI + STDM	58 ± 10.0	303 ± 30.4	396 ± 63.8*	254 ± 18.8
CI + Imuvert	52 ± 15.4	269 ± 27.5	314 ± 22.5*	263 ± 20.5

* $p < 0.05$ with respect to CI (control) values.

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4.0 Discussion

At present, it has been proven that endogenous production of IL-1, IL-6, and TNF-α is required to survive acute radiation syndrome and myelosuppression induced by life-threatening whole body irradiation. The synergistic interaction of these three cytokines stimulates production of a cascade of lineage-specific growth factors, including IL-3, GM-CSF, G-CSF, M-CSF, and so on, that are more restricted to hematopoietic effects [60, 61]. In order to better understand the pathogenesis of combined radiation injury, this work evaluated endogenous as well as LPS-stimulated IL-1, IL-6, and TNF-α production following total body irradiation and combined injury.

Macrophages can be heterogeneous for the production of TNF, IL-1, and IL-6 according to their different sources [66]. In this study we examined the peritoneal, splenic, and bone marrow macrophages to determine possible peculiarities of cytokine production by the different populations of macrophages following radiation, burn, and combined injury. The evaluation of cytokine production in the hematopoietic organs in mice (bone marrow, and spleen) is especially important because these organs contain target cells both for multi-functional cytokines and for lineage-specific hemopoietic growth factors.

Endogenous (non-LPS-stimulated) production of IL-1β was not revealed in any of the culture supernatants of peritoneal, splenic, and bone marrow macrophages following radiation, burn, and combined injury.

LPS-stimulated peritoneal macrophages revealed increased IL-1 production at 72 hr after exposure. It was shown that there were no significant differences of increased IL-1 responses of peritoneal macrophages to radiation versus combined injury (p > 0.1). It should be stressed that cytokine response to radiation exposure was below ELISA sensitivity if macrophages were harvested from the hematopoietic organs in mice. Neither radiation alone nor combined injury enhanced production of IL-1 by in vitro LPS-stimulated and bone marrow macrophages.

It is known that irradiation induces an early increasing of splenic cytokine gene expression for IL-1β determined by RT-PCR [78]. The IL-1β mRNA level in spleen cells increased for a short time prior to regeneration of the spleen [31, 57]. Ionizing radiation also induces an accumulation of IL-1α mRNA in the mouse spleen. An increase in the level of IL-1α protein was observed simultaneously, although the magnitude of increased protein expression did not complement the magnitude of the accumulation of the mRNA [2]. Protein levels of IL-1β in bone marrow and spleen did not appear to change in concert with mRNA levels [10]. These published data agree with our results that macrophages, harvested from bone marrow and spleen within the first three days after radiation alone or combined injury, did not reveal any elevation of IL-1 production as an essential molecular master switch for secretion of IL-3, GM-CSF, G-CSF, and other lineage-specific growth factors.

All three populations of macrophages studied did not produce detectable levels of TNF-α following radiation, burn, and combined injury. The revelation of *in vitro* TNF production is difficult if various preliminary *in vivo* induction procedures were not carried out. Priming has classically been induced in vivo by

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bacteria such as Bacillus Calmette-Guerin or *Corynebacterium parvum*. Within the framework of this study, only non-primed resident macrophages were harvested. This may be one of the possible explanations that LPS-stimulated TNF-α production was not revealed.

TNF-α mRNA expression consistently increased in splenocytes of burned mice at intervals from 4 to 21 days after burn. But in vitro TNF-α production from cultured LPS-stimulated splenic adherent cells of mice was not elevated in the early days after severe thermal injury. It was significantly enhanced from day 6 [64, 73]. TNF production by LPS-stimulated splenic macrophages increased at post burn day 8 versus control (no burn), as well as versus post burn days 1 and 3. By post burn day 8 tissue macrophages may have become primed to respond to a stimulus such as LPS [92]. TNF-α mRNA level was unaltered within the first day after irradiation compared with unirradiated control mice [78]. Therefore, enhancing of TNF-α production by macrophages probably could be observed at later periods after burn, radiation, and combined injury. We regard it as a more acceptable explanation of the revealed fact that LPS-stimulated TNF-α production was not enhanced following radiation, burn, and combined injury.

There are contrary published data to the effect that at early times after thermal injury guinea pig and rat bone marrow macrophages produced different amounts of TNF-α compared with unburned animals [67, 70]. At 2 or 24 h postburn guinea pig mesenteric lymph node and splenic macrophages, primed in vitro by LPS, produced different and sometimes large amounts of TNF-α and IL-6 compared to controls [25]. These data do not coincide with our results because of the differences in the burn models used. The authors of these publications used a more severe guinea pig and rat burn model of 30% total body surface area; we used a murine model with a comparatively light degree burn of 10% TBSA.

Besides that, there may be other explanations connected with cell culture conditions. In murine macrophages, TNF-α production appears to commence almost immediately after exposure to LPS, continues for several hours, peaks at 3 to 4 hr, then ceases abruptly. The reasons for this decrease in TNF-α activity is not clear at present. TNF-α itself can be absorbed by the macrophages. Incubation of murine macrophages in carbon dioxide results in reversible inhibition of LPS-stimulated TNF-α release. The molecule may simply be lost from solution by binding to the tissue culture plate (incubation-reduction phenomenon). Recombinant human TNF-α itself lost activity when incubated more than 12 hr. The mean media concentration of TNF-α activity at 24 hr was significantly less than the mean concentrations at 0, 3, 6, and 12 hr [28, 37, 54, 82, 90]. It is possible that macrophages, stimulated by LPS for 24 hr, may have produced more TNF than macrophages incubated for shorter time periods, but TNF-α loses activity when present in vitro under tissue culture conditions for greater than 12 hr. In our work, macrophages were LPS-stimulated in vitro for 24 hr because that time-period was the most acceptable for simultaneous evaluation of IL-1, IL-6, and TNF-α production in one common supernatant sample [18, 25, 66].

Peritoneal macrophages, incubated with or without addition of LPS to the culture medium, revealed constant two-phase enhancing of IL-6 production following radiation and combined injury. A thermal burn given to irradiated mice did not alter the radiation-induced increase of IL-6 production levels.

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Radiation alone and combined injury did not enhance in vitro production of IL-6 by splenic macrophages (both unstimulated and LPS-stimulated). The levels of cytokine in culture supernatants were lower than ELISA sensitivity limits. These results are in accordance with data that, in spite of increased radiation induced splenic cytokine gene expression for IL-6, no increased protein levels were detected [10].

IL-6 was also not detected in the culture medium of non-stimulated and LPS-stimulated bone marrow macrophages following combined injury at 3, 6, 24, 48, and 72 hr. At the same time, bone marrow macrophages harvested from only irradiated mice revealed an increased IL-6 production at 72 hr after exposure. IL-6 is an important mediator of both IL-1-induced and TNF-induced hematopoietic recovery. The detected differences between radiation alone and combined injury, determined at the local level in the bone marrow, may be considered as unfavorable factors of combined injury pathogenesis.

The results suggest that, in general, peritoneal, splenic, and bone marrow macrophages from combined injury animals did not revealed significant differences in the capacity to produce IL-1β, TNF-α, and IL-6 compared with irradiated only mice (with the exception of the foregoing single fact).

The evaluation of serum cytokine response following radiation, thermal or combined injuries included determination of IL-1β, TNF-α, IL-6, IL-3, and GM-CSF levels. Our study revealed that cytokine levels in the sera of normal healthy mice were below the limits of detection by the enzyme-linked immunosorbent assay (ELISA). These results coincide with the results of many other investigators [10, 22, 50, 51, 59].

The absolute majority of irradiated mice did not reveal detectable amounts of IL-1β, TNF-α, GM-CSF, and IL-3 in their serum samples at 3, 6, 24, 48 and 72 hours after exposure to a minimal lethal dose of 7 Gy. As shown earlier [9, 10], no increased serum GM-CSF, IL-1β, IL-6, and TNF-α levels were detected from days 2 to 14 in irradiated mice. Recently the first work was published that demonstrated an increasing of serum TNF-α levels after exposure to ionizing radiation [33]. However, this work was performed in irradiated rats (versus mice of all foregoing references, and our own experiments), animals were irradiated at a higher dose (10 Gy), and cytokine levels were determined only at the early points of time (15, 60, and 120 min after irradiation).

In the murine burn model that was used for this work, we did not find an elevation of serum IL-1β, TNF-α, GM-CSF, and IL-3. Our results conform to published data. In particular, no significant changes in IL-1, and IL-3 were noted after severe trauma [3, 29]. The presence of serum cachectin was not universally observed in any group of burned animals, even if rats received 30% full-thickness scald burns or were burned and infected [50]. Thermal injury caused a decrease in TNF-α mRNA, and TNF-α could not be found in the serum post burn day 1, or on the day of admission in patients with thermal injuries [17, 49, 93]. In the early period after injury (including the period of burn shock) only 24 patients of 42 had detectable TNF-alpha levels in their plasma. An evident increase of serum TNF activity was revealed only in severely burned patients during burn wound surgical revision, in patients with complications including multiple organ failure and sepsis [40 – 43].

The evaluation of serum cytokine responses following combined injuries, as well as following radiation alone, and thermal burn alone, did not reveal detectable amounts of IL-1β, TNF-α, IL-3, and GM-CSF in the tested serum samples.

At the same time, combined injury induced significant increase of serum IL-6 concentration that was more prolonged versus burn-induced cytokine elevation, and was much higher versus IL-6 levels detected among the irradiated mice. Elevation of serum IL-6 levels after combined injury probably reflects the development of a systemic post-traumatic response.

There are actually several publications that describe increasing serum IL-6 levels in murine or rat burn models [4, 23, 72], and in burned patients [3, 29, 36, 80, 92]. All non-surviving burned patients had higher IL-6 levels than those of surviving patients. IL-6 concentration was highest during the first week after injury and declined over time. These findings suggest that IL-6 may influence metabolic and immunologic responses in the first few weeks following thermal injury [17].

If the data regarding the participation of TNF-α and/or IL-1 in the response to trauma are less consistent, IL-6 has been shown to be elevated in patients or in animals with induction of acute-phase proteins and procoagulation [94]. An increased expression of IL-6 mRNA in liver tissue from rats at 24 h after thermal injury was accompanied by an elevation of IL-6 released from cultured Kupffer cells, by increased serum levels of this cytokine, and with an elevated serum level of acute phase protein. Acute phase proteins are produced as a part of the immune response to trauma, and IL-6 plays an important role in acute phase protein induction [93].

Significant elevation in the production of IL-6 is one of the most potent mediators of early post-burn inflammatory and immune dysfunction. The defects in cellular immune responses in a murine model of thermal injury were mediated in large part by increasing production of IL-6, both systemically and by splenic macrophages [22, 23]. It was shown that treatment with antimurine IL-6 antibody after thermal injury and gavage by E. coli positively affect the outcome during gut-derived sepsis. Survival rate was significantly improved compared with control mice treated with nonspecific IgG or saline. The IL-6 serum concentration was significantly lower after burn and gavage in the treated animals compared with nontreated animals [27].

In the case of combined radiation and thermal injuries it is possible to expect development of all of the aforementioned inflammatory and immunological disorders associated with burns and IL-6 elevation. The demonstrated increase of systemic IL-6 response to combined injury may become self-destructive, and may be capable of explaining (in part) the more severe outcomes of combined injuries compared with acute radiation syndrome alone. For example, enhanced serum IL-6 levels, as shown in this work, may be responsible for the accelerated decrease of leukocytes and platelets in the early phase of peripheral blood damage, as consequences of IL-6-caused procoagulation and inflammatory development.

A comparison of blood cytokine responses with hematological data and survival of mice after combined injury and single BRM injection permits us to offer the following conclusion.

All three BRMs studied did not reveal any effects on the serum IL-3, GM-CSF and TNF-α levels. Only Imuvert injections slightly increased serum concentration of IL-1β in 3-6 hours after combined injury. STDM and heat-killed *L. acidophilus* did not enhance serum IL-1 level. By the way, it has been shown [76], that Betafectin, a novel beta-(1,3)-glucan, has broad-spectrum anti-infective and hematopoietic activities without cytokine induction.

On the other hand, changes of serum IL-6 levels, induced by BRMs, were much more obvious. The IL-6 enhancing capacity of the evaluated BRMs, in terms of absolute values of serum peak levels, was as follows: Imuvert > STDM > LA. In terms of duration of increased serum levels, STDM > Imuvert > LA.

A single injection of "strong IL-6 enhancer" STDM significantly increased endogenous CFU production and caused a more rapid and intensive recovery of bone marrow cellularity and white blood cell count with respect to the control group (combined injury without treatment). At the same time, STDM administration to mice with combined injury resulted in the lowest level of 30-days survival (30% average).

LA, being the "weak(est) IL-6 enhancer", did not increase the number of CFUs, did not improve the recovery of bone marrow cellularity, and did not reveal any significant effect on the leukopenia level and white blood cell recovery rate as compared with control (non-treated combined injury). At the same time, injection of LA resulted in an increase of 30-day survival up to 100%.

It is quite probable that an "inappropriate" acute phase and inflammatory effect as well as procoagulation were induced by abundant serum IL-6 levels after combined injury. Administration of Imuvert or STDM caused further significant elevation of enhanced IL-6 levels that could be responsible for even more evident development of an "inappropriate" effect. All this masks the demonstrated positive effects of BRMs on blood system recovery. As a result, STDM and Imuvert were not very beneficial in providing for mice survival after combined injury. Heat-killed *L. acidophilus*, which demonstrated a weak capacity to enhance increased serum levels of IL-6 after combined injury, promoted survival of mice without improving of hematological status. Recently it was shown [88] that when irradiated mice were treated with IL-11, increased survival of mice was not accompanied by improving hematopoietic status. This suggests that other mechanisms could be involved.

Future investigation should resolve, in greater detail, at least two problems: a) what is the actual role of enhanced serum IL-6 levels in the pathogenesis, the more severe clinical course, and the worse outcomes of combined injury versus acute radiation syndrome, and b) what are the non-hematopoietic activities of heat-killed *L. acidophilus* that promote significant increase of survival following combined injury.

References

1. *Advances In the Treatment of Radiation Injuries*. Eds.: T.J.MacVittie, J.F.Weiss and D.Browne. Advances in the Biosciences, vol. 94, 1996. (Unclassified)

2. Baker W.H. et al. *Comparison of Interleukin-1 Alpha Gene Expression and Protein Levels in Murine Spleen after Lethal and Sublethal Total-body Irradiation*. Rad. Res. 143(3):320-326, 1995. (Unclassified)

3. Bandt J.P. et al. *Cytokine Response to Burn Injury: Relationship with Protein Metabolism*. J. Trauma. 36(5):624-628, 1994. (Unclassified)

4. Bankey P.E. et al. *Interleukin-6 Production after Thermal Injury: Evidence for Nonmacrophage Sources in the Lung and Liver*. Surgery. 118(2):431-438, 1995. (Unclassified)

5. Boudagov R. et al. *Rationale for the Methods of Prevention and Treatment of Toxic Infectious Complications of Combined Radiation and Thermal Injuries*. Medical Radiology. 37(9-10):41-44, 1992. [Russian]. (Unclassified)

6. Boudagov R., Oulianova L. Makarov G. *Preventive Treatment of Combined Radiation Injuries*. In: Proc. Ninth Internat. Congress of Radiation Protection Association. Vienna, Austria. Vol.4. PP. 4-157 - 4-159, 1996. (Unclassified)

7. Boudagov R., Oulianova L. *Therapy of Combined Radiation Injuries with Hemopoietic Growth Factors*. Proc. of the First Int. Conf. The radiological consequences of the Chernobyl accident. Minsk, Belarus. Brussels-Luxembourg, 1996, pp. 641-644. (Unclassified)

8. Boudagov R.S. et al. *Biopreparation Based on Lactobacillus Acidophilus – New Remedy for the Early Therapy of Combined Radiation and Thermal Injuries*. Rad. Biology and Radioecology. 37(5):743-749, 1997 [Russian]. (Unclassified)

9. Chang C.N. et al. *Bone Marrow and Splenic Granulocyte-Macrophage Colony-Stimulating Factor and Transforming Growth Factor-Beta mRNA Levels in Irradiated Mice*. Blood. 86(6):2130-2136, 1995. (Unclassified)

10. Chang C.N. et al. *Sublethal Gamma Irradiation Increases IL-1a, IL-6, and TNF-a mRNA Levels in Murine Hematopoietic Tissues*. J. Interferon and Cytokine Res. 17:567-572, 1997. (Unclassified)

11. Chirigos M.A., Patchen M.L. *Survey of Newer Biological Response Modifiers for Possible Use in Radioprotection*. Pharmac. Ther. 39:243-246, 1988. (Unclassified)

12. *Combined Radiation Injuries: the Pathogenesis, Clinic and Treatment*. Eds. Tsyb A. & Farshatov M. Moscow, Medicine, 1993 [Russian]. (Unclassified)

13. Conklin J.J. et al. *Current Concepts in the Management of Radiation Injuries and Associated Trauma*. Surgery, Gynecology and Obstetrics, 156(6):809-829, 1983. (Unclassified)

14. *Cytokines in Radiation Biology*. Izotoptechnika, Diagnosztika, vol. 37, Suppl., Budapest, 1994. (Unclassified)

15. *Diagnosis and Treatment of Patients with Acute Radiation Syndromes*. Eds: G.Wagemaker and V.G.Bebeshko. European Commission. Luxembourg, 137 p., 1996. (Unclassified)

16. Dijkstra J. et al. *Incorporation of LPS Diminishes its Ability to Induce Tumoricidal Activity and Tumor Necrosis Factor Secretion in Murine Macrophages*. J. Leukocyte Biol. 43(5):436-444, 1988. (Unclassified)

17. Drost A.C. et al. *Plasma Cytokines Following Thermal Injury and Their Relationship with Patient Mortality, Burn Size, and Time Postburn*. J. Trauma. 35(3):335-339, 1993. (Unclassified)

18. Faist E. *The Mechanisms of Host Defense Dysfunction Following Shock and Trauma*. In: Current Topics in Microbiology and Immunology. V.216. Pathology of Septic Shock. Ed.: E. T. Rietschel and H. Wagner. Springer, 259-274, 1996. (Unclassified)

19. Farese A.M. et al. *Acceleration of Hematopoietic Reconstitution with a Synthetic Cytokine (SC-55494) after Radiation-Induced Bone Marrow Aplasia*. Blood. 87(2):581-591, 1996. (Unclassified)

20. Farese A.M. et al. *Combination Protocols of Cytokine Therapy with Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor in a Primate Model of Radiation-Induced Marrow Aplasia*. Blood. 82(10):3012-3018, 1993. (Unclassified)

21. Farese A.M. et al. *Combined Administration of Recombinant Human Megakaryocyte Growth and Development Factor and Granulocyte Colony-Stimulating Factor Enhances Multilineage Hematopoietic Reconstitution in Nonhuman Primates After Radiation-Induced Marrow Aplasia*. J. Clinical Investigation. 97(9):2145-2151, 1996. (Unclassified)

22. Faunce D.E. et al. *Acute Ethanol Exposure Prior to Thermal Injury Results in Decreased T-cell Responses Mediated in Part by Increased Production of IL-6*. Shock. 10(2):135-140, 1998. (Unclassified)

23. Faunce D.E. et al. *Glucocorticoids Protect Against Suppression of T Cell Responses in a Murine Model of Acute Ethanol Exposure and Thermal Injury by Regulating IL-6*. J. Leukoc. Biol. 64:724-732, 1998. (Unclassified)

24. Frasca D. et al. *Use of Hematopoietic Cytokines to Accelerate the Recovery of the Immune System in Irradiated Mice*. Experimental Hematology. 25(11):1167-1171, 1997. (Unclassified)

25. Fukushima R. et al. *Time Course of Production of Cytokines and Prostaglandin E2 by Macrophages Isolated After Thermal Injury and Bacterial Translocation*. Circ. Shock. 42(3):154-162, 1994. (Unclassified)

26. Furuse M. et al. *Acceleration of Granulocytic Cell Recovery in Irradiated Mice by a Single Subcutaneous Injection of Heat-Killed*

Lactobacillus Casei Preparation. J. Rad. Res. 38(2):111-120, 1997. (Unclassified)

27. Gennary R., Alexander J.W. *Anti-Interleukin-6 Antibody Treatment Improves Survival During Gut-Derived Sepsis in Time-Dependent Manner by Enchancing Host Defense.* Critical Care Medicine. 23(12):1945-1953, 1995. (Unclassified)

28. Gifford G. E., Flick D. A. *Natural Production and Release of Tumor Necrosis Factor.* Ciba Found. Symp. 131:3-20, 1987. (Unclassified)

29. Grzelak I. et al. *Surgical Trauma Evokes a Rise in the Frequency of Hematopoietic Progenitor Cells and Cytokine Levels in Blood Circulation.* Europ. Surg. Res. 30(3):198-204, 1998. (Unclassified)

30. Inoue H. et al. *Alpha-Galactosylceramide (AGL-517) Treatment Protects Mice from Lethal Irradiation.* Experimental Hematology. 25(9):935-944, 1997. (Unclassified)

31. Ishihara H. et al. *Immediate-Early, Transient Induction of Interleukin-1 Beta Gene in Mouse Spleen Macrophages by Ionizing Radiation.* J. Rad. Res. 36(2):112-124, 1995. (Unclassified)

32. Jovcic G. et al. *The Effect of IL-1 Receptor Antagonist on the Proliferation of Hematopoietic Progenitor Cells in Regenerating Bone Marrow.* Leukemia. 10(3):564-569, 1996. (Unclassified)

33. Kandasamy S.B. *Possible Involvement of Tumor Necrosis Factor Alpha in Radiation-Induced Hyperthermia in Rats.* Rad. Res. 149:27-31, 1998. (Unclassified)

34. Kawakami M. et al. *Measurement of Interleukin-6, Interleukin-10, and Tumor Necrosis Factor-Alpha Levels in Tissues and Plasma After Thermal Injury in Mice.* Surgery. 121(4):440-448, 1997. (Unclassified)

35. Khlopovskaya E. et al. *Application of Drugs of Protozoan Origin as Means of Prophylaxis and Early Therapy in Combined Radiation-Thermal Injuries.* Science Academy's news. Biological series. 2:227-234, 1993. [Russian]. (Unclassified)

36. Kowal-Vern A. et al. *Interleukin-2 and Interleukin-6 in Relation to Burn Wound Size in the Acute Phase of Thermal Injury.* J. Amer. College of Surgeons. 178(4):357-362, 1994. (Unclassified)

37. Kunkel S.L. et al. *Prostaglandin E2 Regulates Macrophage-Derived Tumor Necrosis Factor Gene Expression.* J. Biol. Chemistry. 263(11):5380-5384, 1988. (Unclassified)

38. Ledney G.D. et al. *Therapy of Infections in Mice Irradiated in Mixed Neutron/Photon Fields and Inflicted with Wound Trauma: a Review of Current Work.* Rad. Res. 128:S18-S28, 1991. (Unclassified)

39. Legeza V.I. et al. *Experimental Study of IL-1b Efficacy in Radiation Injury.* Hematology and Transfusiology, 3:10-13, 1995. [Russian]. (Unclassified)

40. Liu X.S. et al. *A Preliminary Exploration of the Relationship Between Tumour Necrosis Factor (TNF) and Monocytic In Vitro Pro-*

duction of Interleukin-1 (IL-1) and Internal Organ Dysfunction in Severely Burned Patients. Burns. 21(1):29-33, 1995. (Unclassified)

41. Liu X.S. et al. The Significance of Changes in Serum Tumour Necrosis Factor (TNF) Activity in Severely Burned Patients. Burns. 20(1):40-44, 1994. (Unclassified)

42. Ljunghusen O. et al. Endotoxemia and Complement Activation After Severe Burn Injuries - Effect on Leukocytes, Soluble Selectins, and Inflammatory Cytokines. Inflammation. 20(3):229-241, 1996. (Unclassified)

43. Ljunghusen O. et al. Transient Endotoxemia During Burn Wound Revision Causes Leukocyte Beta 2 Integrin Up-Regulation and Cytokine Release. Inflammation. 19(4):457-468, 1995. (Unclassified)

44. MacVittie T.J. et al. Therapeutic Efficacy of Recombinant Interleukin-6 (IL-6) Alone and Combined with Recombinant Human IL-3 in a Non-human Primate Model of High-Dose, Sublethal Radiation-Induced Marrow Aplasia. Blood. 84(8):2515-2522, 1994. (Unclassified)

45. Madonna G.S. et al. Treatment of Mice with Sepsis Following Irradiation and Trauma with Antibiotics and Synthetic Trehalose Dicorinomycolate (S-TDCM). J. Trauma. 31(3):316-325, 1991. (Unclassified)

46. Madonna G.S. et al. Trehalose Dimycolate Enhances Resistance to Infection in Neutropenic Animals. Infection and Immunity. 57(8):2495-2501, 1989. (Unclassified)

47. Makidono R., Makidono Y. Hematopoietic and Lymphoid Cell Recovery from Radiation Damage by Cytokines. Nippon Acta Radiologica. 54(13):1294-1305, 1994. (Unclassified)

48. Mandel L. et al. Treatment of Radiation Disease by Nocardia Fraction: Possible Effect of Inflammatory Cytokines. Int. J. Immunopharmacology. 16(5-6):481-485, 1994. (Unclassified)

49. Mandrup-Poulsen T. et al. Circulating Interleukin-1 Receptor Antagonist Concentrations are Increased in Adult Patients with Thermal Injury. Crit. Care Medicine. 23(1):26-33, 1995. (Unclassified)

50. Marano M.A. et al. Cachectin/TNF Production in Experimental Burns and Pseudomonas Infection. Arch. Surg. 123:1383-1388, 1988. (Unclassified)

51. Matsumoto T. et al. Effect of Interleukin-10 on Gut-Derived Sepsis Caused by Pseudomonas Aeruginosa in Mice. Antimicrobial Agents and Chemotherapy. 42(11):2853-2857, 1998. (Unclassified)

52. McChesney et al. Trehalose Dimycolate Enhances Survival of Fission Neutron-Irradiated Mice and Klebsiella Pneumoniae-Challenged Irradiated Mice. Rad. Res. 121:71-75, 1990. (Unclassified)

53. Milenkovic P. et al. The In Vivo Effect of Recombinant Human Interleukin-1 Receptor Antagonist on Spleen Colony Forming Cells After Radiation Induced Myelosuppression. European Cytokine Network. 6(3):177-180, 1995. (Unclassified)

26

54. Morris D. D. et al. *Endotoxin Induced Tumor Necrosis Factor Activity Production by Equine Peritoneal Macrophages.* Circ. Shock. 30(3):229-236, 1990. (Unclassified)

55. Neelis K.J. et al. *A Single Dose of Thrombopoietin Shortly After Myelosuppressive Total Body Irradiation Prevents Pancytopenia in Mice by Promoting Short-Term Multilineage Spleen-Repopulating Cells at the Transient Expense of Bone Marrow-Repopulating Cells.* Blood. 92(5):1586-1597, 1998. (Unclassified)

56. Neelis K.J. et al. *Simultaneous Administration of TPO and G-CSF After Cytoreductive Treatment of Rhesus Monkeys Prevents Thrombocytopenia, Accelerates Platelet and Red Cell Reconstitution, Alleviates Neutropenia, and Promotes the Recovery of Immature Bone Marrow Cells.* Experimental Hematology. 25(10):1084-1093, 1997. (Unclassified)

57. Nemoto K. et al. *Expression of IL-1 Beta mRNA in Mice After Whole Body X-Irradiation.* J. Rad. Res. 36(2):125-133, 1995. (Unclassified)

58. Neta R. et al. *Inhibition of C-Kit Ligand/Steel Factor by Antibodies Reduces Survival of Lethally Irradiated Mice.* Blood. 81(2):324-327, 1993. (Unclassified)

59. Neta R. et al. *Role of Interleukin 6 (IL-6) in Protection from Lethal Irradiation and in Endocrine Responses to IL-1 and Tumor Necrosis Factor.* J. Exper. Med. 175:689-694, 1992. (Unclassified)

60. Neta R. *Modulation of Radiation Damage by Cytokines.* Stem cells. 15: Suppl.2, 87-94, 1997. (Unclassified)

61. Neta R., Durum S.K. *Whole-Organism Responses to DNA Damage. Modulation by Cytokines of Damage Induced by Ionizing Radiation.* From: DNA damage and repair, Vol.2: DNA repair in higher eukaryotes. Edited by: J.A.Nickoloff and M.F.Hoekstra. Humana Press Inc., Totowa, NY. 587-601, 1998. (Unclassified)

62. Nomoto K. et al. *Radioprotection of Mice by a Single Subcutaneous Injection of Heat-Killed Lactobacillus Casei After Irradiation.* Rad. Res. 125(3):293-297, 1991. (Unclassified)

63. Nothdurft W. et al. *Acceleration of Hemopoietic Recovery in Dogs After Extended-Field Partial-Body Irradiation by Treatment with Colony-Stimulating Factors: rhG-CSF and rhGM-CSF.* Int. J. Rad. Oncology Biology Physics. 37(5):1145-1154, 1997. (Unclassified)

64. O'Riordain D.S. et al. *Molecular Mechanisms of Decreased Interleukin-2 Production after Thermal Injury.* Surgery. 114(2):407-414, 1993. (Unclassified)

65. Ogle C.K. et al. *Effect of Hepatocytes from Normal or Endotoxin Treated Animals on the Production of Tumour Necrosis Factor, Interleukin-6, and Prostaglandin-E2 by Macrophages Incubated In Vitro with Various Fatty Acids.* Europ. J. Surgery. 161(2):123-127, 1995. (Unclassified)

66. Ogle C.K. et al. *Heterogeneity of Kupffer Cells and Splenic, Alveolar,*

and Peritoneal Macrophages for the Production of TNF, IL-1, and IL-6. Inflammation. 18(5):511-523, 1994. (Unclassified)

67. Ogle C.K. et al. *Production of Cytokines and PGE2 and Cytotoxicity of Stimulated Bone Marrow Macrophages After Thermal Injury and Cytotoxicity of Stimulated U-937 Macrophages.* Inflammation. 17(5):583-594, 1993. (Unclassified)

68. Ogle C.K. et al. *Production of Cytokines and Prostaglandin E2 Subpopulations of Guinea Pig Enterocytes: Effect of Endotoxin and Thermal Injury.* J. Trauma. 41(2):298-305, 1996. (Unclassified)

69. Ogle C.K. et al. The 1994 Linberg Award. *The Production of Tumor Necrosis Factor, Interleukin-1, Interleukin-6, and Prostaglandin E2 by Isolated Enterocytes and Gut Macrophages: Effect of Lipopolysaccharide and Thermal Injury.* J. Burn Care & Rehabilitation. 15(6):470-477, 1994. (Unclassified)

70. Ogle C.K. et al. *The Activation of Bone Marrow Macrophages 24 Hours After Thermal Injury.* Archives of Surgery. 128(1):96-100, 1993. (Unclassified)

71. Ogle C.K. et al. *Thermal Injury Induces the Development of Inflammatory Macrophages from Nonadherent Bone Marrow cells.* Inflammation. 21(6):569-582, 1997. (Unclassified)

72. Ohzato H. et al. *Systemic Production of Interleukin-6 Following Acute Inflammation.* Biochem. & Biophys. Res. Communications. 197(3):1556-1562, 1993. (Unclassified)

73. Oriordain M.G. et al. *Dosage and Timing of Anti-TNF-Alpha Antibody Treatment Determine Its Effect on Resistance to Sepsis After Injury.* J. Surgical Research. 64(1):95-101, 1996. (Unclassified)

74. Oulianova L. P. et al. *The Study of the Effect of Interleukin-1b Against the Combined Radiation-Thermal Injuries.* Rad. Biology. Radioecology. 37(2):175-181, 1997 [Russian]. (Unclassified)

75. Patchen M. L. et al. *Effects of Combined Administration of Interleukin-6 and Granulocyte Colony-Stimulating Factor on Recovery from Radiation-Induced Hemopoietic Aplasia.* Experimental Hematology. 21(2):338-344, 1993. (Unclassified)

76. Patchen M. L. et al. *In Vitro and In Vivo Hematopoietic Activities of Betafectn (R) PGG-Glucan.* Experimental Hematology. 26(13):1247-1254, 1998. (Unclassified)

77. Peng D. Z., Huang W. H., Li A. *The Roles of Macrophages in Immune Dysfunction Following Severe Thermal Injury.* Chinese J. Surgery. 32(5):310-313, 1994. (Unclassified)

78. Peterson V. M. et al. *Gene Expression of Hematoregulatory Cytokines is Elevated Endogenously after Sublethal Gamma Irradiation and is Differently Enhanced by Therapeutic Administration of Biological Response Modifiers.* J. immunology. 153:2321-2330, 1994. (Unclassified)

79. Renz et al. *Release of Tumor Necrosis Factor-α from Macrophages.*

Enhancement and Suppression are Dose-Dependently Regulated by Prostaglandin E2 and Cyclic Nucleotides. J. Immunology. 141(7):2388-2393, 1988. (Unclassified)

80. Rodriguez J.L. et al. *Correlation of the Local and Systemic Cyto-kine Response with Clinical Outcome Following Thermal Injury.* J. Trauma. 34(5):684-694, 1993. (Unclassified)

81. Rogacheva S.A. et al. *Study of the Antiradiation Effect of Interleukin-1b in Experiments.* Radiat. Biology. Radioecology. 34(3):419-423, 1994. [Russian]. (Unclassified)

82. Sayers T.J. et al. *The Production of Tumor Necrosis Factor by Mouse Bone Marrow-Derived Macrophages in Response to Bacterial Lipopolysaccharide and a Chemically Synthesized Monosaccharide Precursor.* J. Immunol. 38(9):2935-2940, 1987. (Unclassified)

83. Sureda A. et al. *Granulocyte Colony-Stimulating Factor Admin-istered as a Single Intraperitoneal Injection Modifies the Lethal Dose95/30 in Irradiated B6D2F1 Mice.* Haematologica, 83(9):863-864, 1998. (Unclassified)

84. *The Pathogenesis and Treatment of Combined Radiation-Thermal Injuries.* Eds. Tsyb A. & Britun A. Moscow, Medicine, 1989 [Rus-sian]. (Unclassified)

85. *The Pathophysiology of Combined Injury and Trauma. Management of Infectious Complications in Mass Casualty Situation.* Eds. Gruber D. et al. Orlando, Florida, Academic Press, Inc., 1987. (Unclassified)

86. Tsuneoka K. et al. *Timing of Administration of Heat-Killed Lactoba-cillus Casei Preparation in Mice.* J. Rad. Res. 35(3):147-156, 1994. (Unclassified)

87. Tsyb A., Boudagov R., Oulianova L. *Experimental Studies to Im-prove Diagnosis and Treatment of Combined Radiation/Thermal Injuries. In: Diagnosis and Treatment of Patients with Acute Radia-tion Syndromes.* JSP No 3. Final report. Brussels-Luxembourg. PP. 82-91, 1996. (Unclassified)

88. Van Der Meeren et. al. *Hematological Status and Survival Data from Lethally Irradiated Mice Treated with IL-11. Diagnosis and Treat-ment of Radiation Injury.* International Conference de Doelen Rot-terdam. Book of abstracts. 1998. (Unclassified)

89. Van Der Meeren et. al. *Improvement of Survival and Hematopoietic Recovery with TPO Following High Doses of γ Irradiation. Diagno-sis and Treatment of Radiation Injury.* International Conference de Doelen Rotterdam. Book of abstracts. 1998. (Unclassified)

90. West M. A. et al. *Acetazolamide Treatment Prevents In Vitro Endo-toxin-Stimulated Tumor Necrosis Factor Release in Mouse Macro-phages.* Shock. 10(6):436-441, 1998. (Unclassified)

91. Wu D. D. et al. *Hematopoietic Cytokines Enhance Survival of SCID Mice Undergoing High-Dose Irradiation.* Experimental Hematology. 22(2):202-207, 1994. (Unclassified)

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